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LIPOLYTIC POTENTIALS OF THREE BACTERIAL STRAINS ISOLATED FROM AQUACULTURE BENTHOS IN EAST KOLKATA WETLANDS, INDIA

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ABSTRACT: Prokaryotic species in the soil microenvironment perform a variety of activities in the circulation of nutrients by breaking down complex detritus molecules into simpler ones by the production of extracellular enzymes. In this study, three bacterial strains were isolated from benthic soil of an aquaculture farm in East Kolkata Wetlands, India, with considerable lipolytic potential. Upon molecular characterization using 16S rDNA analysis the strains were identified as *Enterobacter cloacae* Strain P4cR1, *Klebsiella pneumoniae* Strain P5aR1, and *Serratia marcescens* Strain P5bR1. Enzymatic analyses revealed that *S. marcescens* Strain P5bR1 shows the best enzymatic activity with 16.82±0.87 IU $ml^{-1}min^{-1}$ followed by *K. pneumoniae* Strain P5aR1 with 12.53±1.84 IU $ml^{-1}min^{-1}$ and *E. cloacae* Strain P4cR1 showing the lowest activity at 9.48±1.05 IU $ml^{-1}min^{-1}$. The enzymes were stable at a pH range of 7 to 8 and a temperature range within 30 to 40°C. This is probably the first report of bacterial strains with lipolytic potential isolated from the benthic soil of an aquaculture farm. These bacteria may find their uses not only in bioremediation purposes but in industrial applications also.

Keywords: Aquaculture benthos, Bacteria, Lipase, pNpp analysis, 16S rDNA.

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1. INTRODUCTION

Bacteria in benthic soil uses complex substances like long-chain fatty acids as the substrate to gain nutrition [1]. These molecules are abundant in the soil sediment as detritus materials. Bacteria produce extracellular lipases to degrade the complex lipid molecules to gain nutrition. Lipase producing bacteria has been reported from different geographical conditions and various ecosystems, like lakes [2], coastal region [3], riverine ecosystems [4], continental shelves undersea [5] and even in the cryosphere of the Arctic region [6,7]. Extracellular lipase producing bacteria has intrigued the scientific community for their uses in the field of bioremediation as well as in the industry. Extracellular lipase producing bacteria finds its application primarily in the field of bioremediation of oil spills [8–10], biodiesel production [11–13], and many industrial uses as well [1,14]. The current study aims to find extracellular lipase producing bacteria from the benthic soil of an aquaculture farm in East Kolkata Wetlands. Though several references exist for lipase producing bacteria from several soil sources, the same from the benthic soil of aquaculture farms are very meager. In East Kolkata Wetlands, a unique method of aquaculture practice is performed known as the "bheri" culture [15]. In this method, the sewage water from municipal sources is stored in shallow ponds and organic fertilizers are added to prepare the pond for aquaculture purposes [16]. However, during the monsoons, these farms receive a huge amount of rainwater runoffs from the adjacent industrial and city roads [15]. Thus a considerable amount of oil spills from automobiles and other industrial effluents are mixed in the water and sludge. These bheris are used as a natural filtration unit employing the horizontal culture of fishes where bacteria and algae act as degraders and fishes act as control organisms in this method [17]. So the presence of bacteria with lipolytic potential is imminent from this bheris. Though there are references of bacteria with Lipolytic potential in East Kolkata Wetlands proper data on the efficacy of the enzyme and use of molecular techniques for the identification of those lipolytic strains are currently unavailable.

2. MATERIALS AND METHODS

Sample Collection

Benthic soil samples were collected from an aquaculture farm in East Kolkata Wetlands, Kolkata, West Bengal, India (Latitude – 22.5699° Longitude- 88.4394°). Soil samples from the top surface of the benthos were aseptically collected from a depth of 60cm of the water column. The samples

were immediately transferred to pre-sterilized polycarbonate containers with a sterile spatula. The samples were marked and taken to the laboratory for further processing.

Isolation and Purification

Bacterial Strains were isolated from soil using the protocol of Vieira and Nahas, 2005, with some modifications. 5gm (wet weight) of soil were first homogenized in sterile water and passed through 2mm filter mesh. The filtrate was then serially diluted up to 10^8 times and 100 µl of 10^4 to 10^8 diluted suspensions were spread on Agar plates (tryptone 1%, yeast extract 1%, Agar 2%) supplemented with tributyrin (0.25%) and Tween 20 (0.25%), as an emulsifier. The plates were incubated at 37℃ for 24 hours. Extracellular lipase produced by these strains degraded tributyrin, thus resulting in the formation of a halo. The diameter of the halo as well as that of the colony were noted. From the supplementary culture, the bacteria were separated based on colony morphology (shape, size, color, transparency, margin contour, surface topology, etc.) and were transferred to a Tryptone Soya Agar (TSA) plate by streaking. Repeated subcultures were done until the pure culture was obtained. The final isolates were routinely cultured in Nutrient Agar.

Biochemical characterization

Cumulatively, 14 biochemical tests were performed according to *Bergey's Manual of Systematic Bacteriology* [19] including determination of Gram Character, Methyl Red (MR), Voges- Proskauer (VP), Triple Sugar Iron (TSI), etc. to determine the biochemical characters of the strains with lipolytic potential.

Molecular characterization

Molecular characterization was done by 16S rDNA analysis using universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'), using standard protocols [20]. The PCR amplicons were purified and sequenced from Xcelris Labs Ltd. (Ahmedabad, India). Standard *In-silico* analyses of the sequenced samples were done. The sequence obtained was subjected to BLASTn analysis (NCBI GenBank Database) to find the nearest neighbors, the sequences of which were downloaded in FASTA format. The sequences were aligned using CLUSTAW and then were subjected to phylogenetic analysis using Neighbor-joining method [21] and Maximum Likelihood Composite model [22]. All *In-silico* analyses were done in MEGA X software [23].

Lipase Activity Assay

For quantitative estimation of the enzymatic activity, the strains selected were first cultured in Nutrient broth overnight. On the following day, 10% of the overnight culture was inoculated in 45ml sterile Minimal Salt medium supplemented with 0.25% tributyrin and were incubated in a shaker incubator at 37℃, 160rpm for 48hours. Harvesting of the crude enzyme extract was done by centrifugation of the culture at 10000rpm for 20 minutes at 4℃. The supernatant was collected and preserved at 4℃ for further studies.

Extracellular lipase activity was measured from the supernatant following the protocol given by Mongkolthanaruk and Dharmsthiti, 2002 [24], with some minor modifications. Hydrolysis of p-nitrophenyl palmitate (pNPP) occurred due to the extracellular lipase in the crude extract forming p – nitrophenol (pNP), a chromogenic substance that shows peak absorption at 405nm. For this process, Solution A (0.062 gms of pNPP in 10ml of 2-propanol, sonicated for 2 minutes) and Solution B (0.4% Triton –X 100, 0.1% gum Arabic in 50ml Tris-HCl, pH 8.0) were prepared. The reaction mixture was prepared by adding 180µl of Solution A with 1.62ml of Solution B and 200µl of the crude extract. The solution was mixed thoroughly and incubated at 30℃ for 20 minutes. After incubation, the absorbance of the solution was measured spectrophotometrically at 405nm. The OD value thus obtained were plotted against a standard curve of pNP prepared with known concentrations. The results were interpreted as One Unit of Lipase activity = 1µmol of pNP released per minute per ml of enzyme. The results were expressed in IU ml^{-1} min^{-1}.

pH and Temperature stability

Cell-free supernatants containing the crude enzymes were tested for their optimal pH and thermostability. The same process of enzymatic analysis was done by varying the pH of the enzyme production media and temperature of the enzyme before incubation. The pH was tested within a range of 4 – 10 (7 samples with an interval of 1 pH) and the temperature was tested from 20°C to 70°C (6 samples with 10°C intervals).

Statistical analysis

Experiments of the enzymatic analysis of lipase were performed in triplicate and values are expressed as mean values ± standard deviation (SD). Statistical analyses were done using GraphPad Prism 7 (GraphPad Software, USA).

3. RESULTS AND DISCUSSION

Isolation, Purification, and Molecular Characterization

Three bacterial strains were isolated with lipolytic potential from the benthic soil sample. Upon purification of samples from Tributyrin-Tween 20 Agar plates, three bacterial strains were isolated and named Strain P4cR1, Strain P5aR1, and Strain P5bR1 respectively. These strains were selected for further downstream analyses.

Biochemical characterization

Detailed biochemical characterization results of the isolates are given in Table 1. The isolates showed a varying degree of fermentation potential in both anaerobic and aerobic conditions. The strains were also able to produce other extracellular enzymes like urease and amylase.

Molecular Characterization

Upon molecular characterization and In-silico analyses, the strains were identified as *Enterobacter cloacae* Strain P4cR1, *Klebsiella pneumoniae* Strain P5aR1, and *Serratia marcescens* Strain P5bR1. The sequence data of the three strains were submitted to the GenBank vide Accession Numbers of

MT279676, MT254946, and MT279682 respectively. The phylogenetic relationship of the three strains is shown with 10 nearest neighbors of each strain obtained from the GenBank in a Neighbor-joining tree in Figure 1. The tree was drawn to scale and are expressed as the number of base substitution per site. A total of 30 nucleotide sequences were analyzed with 968 positions in the final dataset.

Lipase Activity Assay

The screening test result performed in Tributyrin – Tween 20 agar media revealed a glimpse of the comparative account of the lipolytic potential of the strains involved and is shown in Table 2. As revealed by pNpp assay, *S. marcescens* Strain P5bR1 scores the best enzymatic activity with 16.82±0.87 IU $ml^{-1}min^{-1}$ followed by *K. pneumoniae* Strain P5aR1 with 12.53±1.84 IU $ml^{-1}min^{-1}$ and *E. cloacae* Strain P4cR1 showing lowest activity at 9.48±1.05 IU $ml^{-1}min^{-1}$. Results of temperature and pH stability estimation of the enzyme activity of the three isolates are depicted graphically in Figure 3a and 3b respectively. Both the analyses showed a common pattern for both the enzymes. For pH, the maximum activity of the enzymes obtained from the strains showed the highest activity within a range of 7 to 8 and diminishing at either extremity. In the case of temperature, the highest activity was recorded within 30 to 40°C with the same trend of diminishing activity with either increase or a decrease in the temperature.

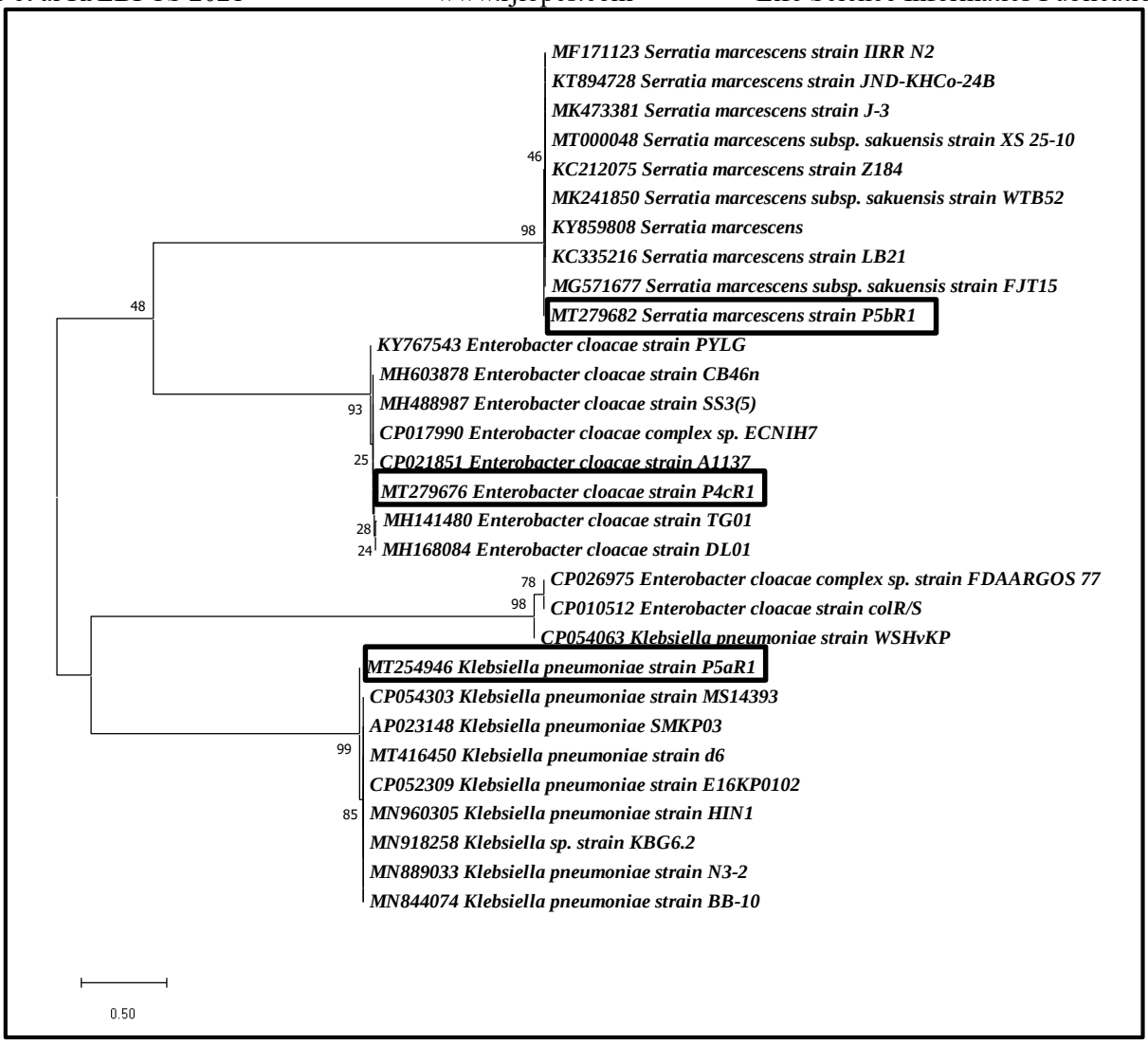

Figure 1: A combined phylogenetic analysis of all the lipase producing strains showing their interrelationship. The tree is drawn to scale using Neighbor-joining method and Maximum Likelihood Composite model.

Table 1: Biochemical test results of the lipase producing strains.

Biochemical Tests		Strain		
		P4cR1	P5aR1	P5bR1
TSI	Slant	Y	Y	Y
	Butt	Y	Y	Y
	H_2S	-	-	-
	Gas	-	+	-
Oxidase		-	-	-
Catalase		+	+	+
Gram Staining		-	-	-
KOH String Test		-	-	-
MR		+	-	-
VP		+	+	-
Gelatinase		-	-	-
Indole		-	-	-
Motility		+	-	+
DNase		-	-	-
MacConkey		W	W	W
Urease		-	+	-
Citrate		-	+	-
Amylase		+	+	+

[+ = Positive result, - = Negative result, Y = Sugar Fermenters (Slant – Anaerobic/ Butt – Aerobic)

R = Negative result (TSI and MacConkey media)

Table 2: The results of screening tests in Tributyrin Agar for extracellular lipase producing isolates. The diameter of the Clearing Zone and that of the colony are shown. Enzymatic activities of extracellular lipase are shown as Mean± SD.

Strain	Diameter of the Clearing Zone (Halo) (in mm)	Diameter of the colony (in mm)	Activity (U $ml^{-1}min^{-1}$)
P4cR1	8.6	3.4	9.48±1.05
P5aR1	14.7	4.5	12.53±1.84
P5bR1	18.6	4.9	16.82±0.87

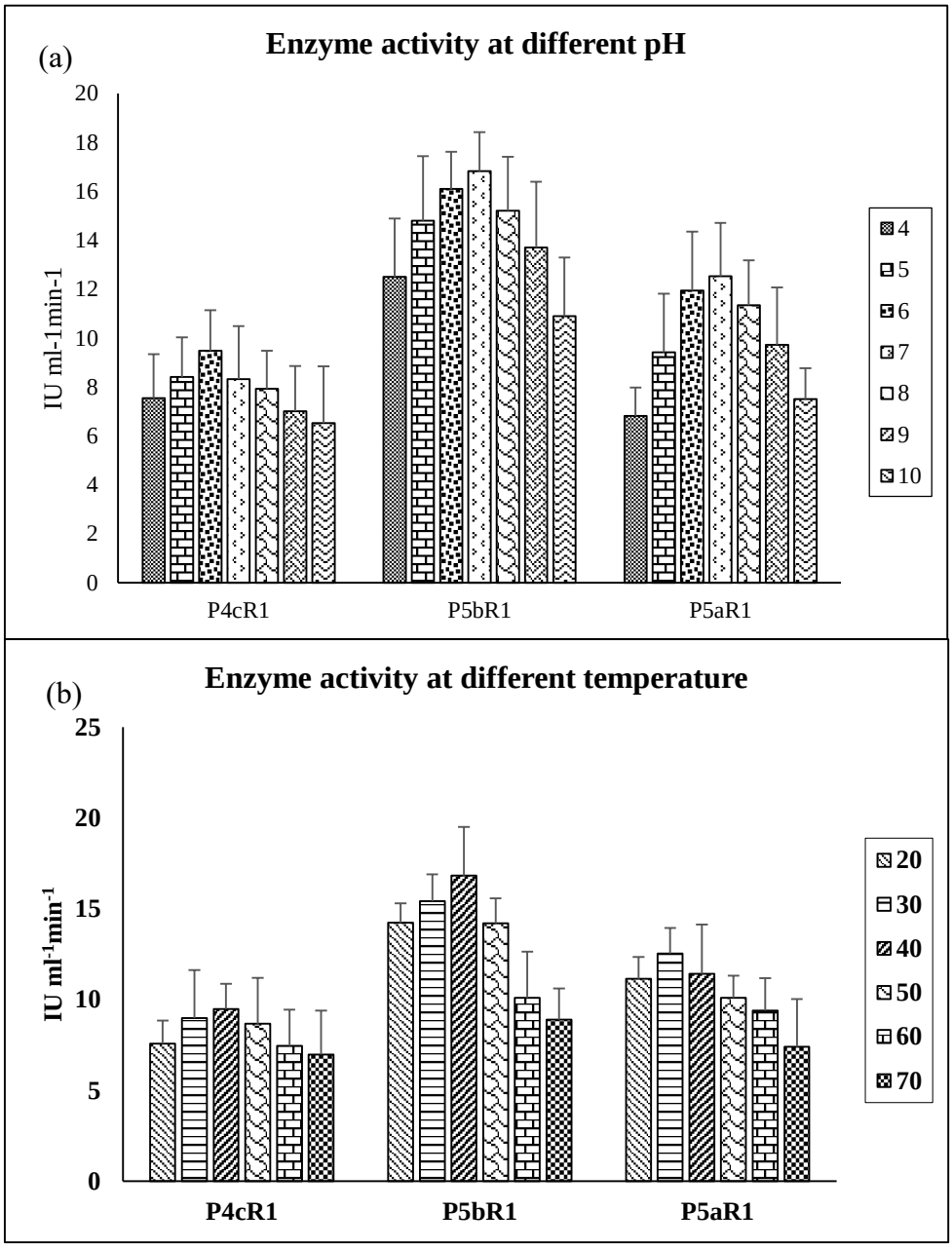

Figure 2: Graphical representation of the variation of activity of extracellular lipase as revealed by pNPP assay with varying pH ranging from pH 4 -10 (a) and temperature ranging from 20°C - 70°C (b).

4. CONCLUSION

Bacterial strains with lipolytic potential are of biotechnological and ecological importance, hence the efficacy of the strains has been widely studied. From the East Kolkata Wetlands region, several genera of bacteria have already been reported [25,26]. However, this is the first report on the efficacy of extracellular lipase producing strains isolated from benthic soil of aquaculture farms in East Kolkata Wetlands. When compared to previously reported articles the efficacy of the extracellular lipase revealed in this study stands at par [4,7,27,28]. Lipase-producing bacteria find its industrial application in biodiesel production [13], as a biocatalyst in several agro and pharmaceutical

industries [29]. In the field of bioremediation, lipase producing bacteria can be used for bioaugmentation purposes in neutralizing hydrocarbon pollution that may arise due to oil spills and industrial effluents [8,9]. Since the extracellular lipase produced by the bacterial strains is stable and shows the most efficacy at a range of 30°C to 40°C, these strains can be one of the potential candidates for biotechnological and bioremediation practices in tropical countries.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

16S rDNA sequences of the bacterial strains are accessible at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) bearing accession numbers MT279676, MT254946, and MT279682

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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